

1 Running head: Bhat et al, Spermatogenesis and Apoptosis in ob/ob Mice

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3 **Influence of a leptin deficiency on testicular morphology, germ cell apoptosis and**
4 **expression levels of apoptosis-related genes in the mouse**

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23 Abstract

24 Leptin deficient (ob/ob) male mice are morbidly obese and exhibit impaired reproductive
25 function. The objective of this study was to assess the effect of a leptin deficiency on
26 testicular morphology, germ cell development, apoptotic activity within germ cells and
27 expression levels of apoptosis-related genes in the testis. Sixteen week-old ob/ob male
28 mice (N = 8) and controls (N = 8) were killed and reproductive organs weighed. Testes
29 were processed for either histomorphological analysis (H&E staining), germ cell
30 apoptosis assessment (TUNEL method), or apoptosis-related gene expression analysis
31 (microarray). Cross sections of the testes of leptin deficient animals showed reduced
32 seminiferous tubule area, fewer pachytene spermatocytes and fewer tubules exhibiting
33 elongated spermatids/mature spermatozoa. Condensation of germ cell nuclei and Sertoli
34 cell vacuolization were evident in the testes of some ob/ob animals. Overall there was an
35 elevation of apoptotic activity in the germ cells of ob/ob mice particularly within the
36 pachytene spermatocytes. With microarray technology, we identified nine pro-apoptosis-
37 related genes that were expressed at a significantly higher level in the testes of ob/ob
38 mice compared to controls. Among these were members of the tumor necrosis factor
39 receptor super family 1A and 5 (TNFR1 and 5) and peptidoglycan recognition proteins
40 (associated with the extrinsic apoptotic pathway), and granzymes A and B, growth arrest
41 and DNA damage inducible 45 gamma, sphingosine phosphate lyase 1 and caspase 9
42 (associated with the intrinsic apoptotic pathway). The results of the current study show
43 that a leptin deficiency in mice is associated with impaired spermatogenesis, increased
44 germ cell apoptosis and up-regulated expression of pro-apoptotic genes within the testes.
45 Key Words: spermatogenesis, seminiferous tubules, testis, cytomorphometry, microarray

46 **Introduction**

47 Previous studies have shown that reproductive function is impaired in the
48 genetically obese (ob/ob) mouse (Jones and Ainsworth-Harrison, 1957; Swerdloff et al,
49 1976). Although the mechanism(s) responsible for the reproductive failure remains far
50 from clear, it has been suggested that the infertility results from impaired hypothalamic
51 GnRH secretion (Swerdloff et al, 1976; Batt et al, 1982). However, leptin receptors have
52 also been identified on germ cells and Leydig cells within the testis (El-Hefnaway et al,
53 2000; Caprio et al, 2003), suggesting that leptin may also play a direct regulatory role in
54 reproduction at the level of the gonad. Leptin replacement therapy, but not food
55 restriction, is associated with a restoration of spermatogenesis and reproductive function
56 (Mounzih et al, 1997); indicating that the morbid obesity is not the cause of the infertility.

57 Mammalian development is tightly regulated by cell proliferation as well as cell
58 death (Ellis et al, 1991; Raff, 1992). Cell death that occurs during embryogenesis,
59 metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover is called
60 programmed cell death or apoptosis (Ellis et al, 1991; Raff, 1992). When the testicular
61 environment is not able to support spermatogenesis, the process of apoptosis decreases
62 the level of proliferation of early germ cells (Lee et al, 1997). Apoptosis within germ
63 cells is characterized by internucleosomal fragmentation of DNA, chromatin
64 condensation, phagocytosis by Sertoli cells, and cell disintegration (Billig et al, 1995). It
65 has been suggested that perhaps as high as 75% of potential mature spermatozoa are
66 eliminated through the apoptotic pathways (Billig et al, 1995).

67 There are at least two major pathways for apoptosis, the extrinsic and intrinsic
68 pathways (Sinha Hikim et al, 2003). The intrinsic pathway involves the release of

69 cytochrome c from the mitochondria into the cytosol resulting in the activation of the
70 initiator caspase 9 and the subsequent activation of the executioner caspases 3, 6 and 7.
71 Caspases are cysteine proteases that mediate specific cleavage events in dying cells.
72 Members of the Bcl-2 family of proteins play a major role governing this mitochondria
73 dependent pathway (Reed, 2000). The Fas-FasL system is involved in the extrinsic
74 apoptotic pathway (Nagata and Golstein, 1995). Fas, a transmembrane receptor protein,
75 and its ligand FasL belong, respectively, to the tumor necrosis factor (TNF) receptor and
76 protein families (Watanabe-Fukunaga et al, 1992; Nagata and Golstein, 1995; Nagata,
77 1997). Binding of FasL to Fas results in the recruitment of FADD (Fas associated death
78 domain) and this complex then activates the initiator caspase, caspase 8. The intrinsic and
79 extrinsic pathways converge on caspase 3 and other executioner caspases that drive the
80 cleavage of various cellular substrates resulting in fragmentation of chromosomal DNA
81 and subsequent formation of apoptotic bodies.

82 The objectives of the current study were to further characterize testicular
83 morphology and spermatogenesis in the leptin deficient mouse and assess the potential
84 involvement of increased germ cell apoptosis in the processes that ultimately alter the
85 fertility of this animal. Microarray technology was also employed to identify potential
86 apoptosis-related genes whose expression levels within the testis are altered by the leptin
87 deficiency.

88 **Materials and Methods**

89 **Animals and tissue preparation**

90 This study was conducted according to the principles and procedures of the
91 National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

92 Sixteen-week old C57BL/6J-Lep(ob) (ob/ob, N=8) and C57BL/6J (littermate controls, N
93 = 8) male mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with halothane
94 vapors and decapitated. At sacrifice, blood was collected, and testes and seminal vesicles
95 were weighed. One testis from each animal was fixed in 10% formalin, paraffin
96 embedded, and sectioned (6-8 μ m) for subsequent hematoxylin-eosin (H&E) staining and
97 TUNEL assay. The other testis from two controls and three ob/ob mice were immediately
98 snap frozen in liquid nitrogen and stored at -70°C for subsequent isolation of total RNA
99 and gene chip microarray.

100 **In situ DNA 3' end labeling of apoptotic cells**

101 The Apop Tag apoptosis detection kit (Serological Co., Norcross, GA) was
102 employed for labeling of DNA fragmentation. The *in situ* terminal deoxynucleotidyl
103 transferase (TdT) mediated by deoxy-UTP-digoxigenin nick end labeling (TUNEL)
104 method was used to localize apoptotic cells in testis sections. Briefly, sections were
105 washed with PBS and pretreated with 20 μ g/ml proteinase K (25°C, 15 min). The
106 sections were then incubated with TdT reaction mixture in a humidified chamber (37°C,
107 60 min), washed with PBS and incubated with anti-digoxigenin antibody conjugated to a
108 rhodamine fluorescent marker in the humidified chamber (25°C, 30 min). Nuclei were
109 counterstained with 0.5 μ g/ml 4', 6-diamino-2-phenylindole, dihydrochloride (DAPI).
110 For negative staining controls, the TdT reaction mixture was omitted.

111 **Hormone assay**

112 Serum samples were assayed for total leptin and testosterone using commercially
113 available ELISA kits (Assay Designs, Ann Arbor MI; Alpha Diagnostic International,
114 San Antonio, TX, respectively). All samples were run in duplicate in the same assay.

115 The minimum detection limits for the leptin and testosterone assays were 5 and 10 pg/ml,
116 respectively. The intraassay coefficient of variations for leptin and testosterone were 16%
117 and 6%, respectively.

118 **Histology and histomorphometry of testes**

119 Hematoxylin - eosin (H&E) stained testes sections from two control and two
120 ob/ob animals were selected for detailed morphological analysis under light microscopy.
121 H&E stained testicular sections from six ob/ob and six control mice were also used to
122 assess the effect of a leptin deficiency on seminiferous tubule area (profile over the
123 periphery of all the cross-sectioned tubules), number of spermatocytes per cross-
124 sectioned tubule, and percentage of cross sectioned tubules exhibiting sperm bundles
125 (elongated spermatids and spermatozoa). Imaging technology (ImagePro Plus Software,
126 Media Cybernetics, Silver Spring, MD) was employed for these measurements.

127 The number of TUNEL positive germ cells was counted in ten random fields from
128 the testicular cross-sections of five ob/ob and five control animals, and the average
129 number of TUNEL positive cells per tubule and the total number of TUNEL positive
130 cells for the 10 fields were calculated for both the ob/ob and control groups.

131 **Microarray sample preparation and hybridization**

132 Total RNA from the testes of ob/ob (N=3) and controls (N=2) was extracted with
133 TRIzol Reagent (Life Technologies, Rockville, MD), cleaned (RNAqueous kit, Ambion,
134 Austin, TX) and converted to double-stranded cDNA (Invitrogen, Superscript Choice
135 System, Carlsbad CA) using a T7-(dT)₂₄ primer. The double-stranded cDNA was
136 cleaned using phase Lock Gels (Eppendorf, Westbury, NY), and an RNA transcript
137 labeling kit (Enzo Diagnostics, Farmingdale, NY) was used to synthesize cRNA. Biotin

138 labeled cRNA was cleaned (GeneChip Sample Cleanup Module, Affymetrix Inc, Santa
139 Clara, CA) and quantified spectrophotometrically. 20 µg of the *in vitro* transcription
140 product was fragmented in fragmentation buffer at 94°C for 35 min. After fragmentation,
141 15 µg of the biotinylated cRNA was hybridized to an Affymetrix Murine Genome
142 U74AV2 GeneChip at 45°C for 16 h, washed, stained with streptavidin phycoerythrin,
143 and scanned according to manufacturer's guidelines.

144 **Microarray data processing**

145 Data analysis was performed by Affymetrix Microarray Suite (MAS) 5.0
146 software. The microarray suite references the experimental file to select an analysis
147 algorithm for a cell intensity file that generates a gene chip file. Single array analysis was
148 used to build the databases of gene expression profiles. Affymetrix GCOS software was
149 used to normalize and analyze the data. Detection P-value (set at $P < 0.05$) was used to
150 statistically determine whether a transcript is expressed on the chip. The software
151 generated a present (P), marginal (M) or absent (A) call for each transcript based on the
152 P-value. To obtain differentially expressed genes for each condition, Affymetrix gene
153 chip software was used to compare each of the ob/ob testes arrays to that of the control
154 arrays. Absolute calls (present, marginal, and absent) and the average difference (RNA
155 abundance) for each gene were then imported into Genespring software (Silicon
156 Genetics, Redwood City, CA) for further analysis. By combining the fold change and the
157 present calls derived from the comparisons, we obtained a list for each condition.
158 Differential expression was calculated as the increase between the two conditions, i.e.,
159 ob/ob testes versus controls. A gene was considered differentially expressed when the
160 standard deviation of the signal increase or decrease was significantly smaller than the

161 absolute change in average difference and the calculated confidence level of a gene was
162 set greater than 95% (P value < 0.05 based on unpaired t-test). A general view of the
163 effect of the leptin deficiency on gene expression in the testes was obtained by Self
164 Organizing Map (SOM) cluster analysis using Genespring software (Silicon Genetics) on
165 replicate samples. Selected clusters were examined for biological function and pathway
166 analysis using Affymetrix Netfix Analysis Center (<http://www.affymetrix.com>). Netfix
167 detailed and annotated individual probe sets based on biological and molecular function
168 or cellular localization using the Gene Ontology public database.

169 **Statistical analysis**

170 One-way ANOVA was used to compare the effects of a leptin deficiency on body
171 and organ weights, serum leptin and testosterone concentrations, seminiferous tubule
172 area, number of spermatocytes, percent tubules with sperm bundles, total number of
173 TUNEL positive germ cells and the number of apoptotic germ cells/tubule between testes
174 from ob/ob and control animals. P values <0.05 were considered as significant for the
175 differences observed between the ob/ob and control animals. Data are presented as the
176 mean \pm SEM.

177 **Results**

178 **Body and organ weights and hormone concentrations**

179 Body weights in the leptin deficient mice were significantly increased while the
180 weights of the testes (P<0.05) and seminal vesicles (P<0.05) were reduced in ob/ob
181 animals compared to control animals (Table 1). Serum leptin concentrations were at or
182 below detection limits in leptin deficient mice. Testosterone concentrations in ob/ob mice
183 did not differ statistically from control animals.

184 **Histology and histomorphometry of testes**

185 Light microscopy of the testes of ob/ob mice suggested that spermatogenesis was
186 impaired. There was evidence of increased germ cell degeneration and condensation of
187 germ cell nuclei (Figure 1; comparing Panels B and C with Panel A). Sertoli cell
188 vacuolization was also observed in leptin deficient mice, and some of the Leydig cells of
189 the ob/ob testes had an abnormal fibroblast-like appearance. The effect of the leptin
190 deficiency on testicular morphology was not universal. While some of the tubules of
191 ob/ob animals appeared to be comprised of only Sertoli cells and without a clear lumen,
192 there were regions in which tubular morphology and spermatogenesis appeared normal
193 (Figure 1, Panel D). Occasionally, multinuclear giant cells were encountered in the center
194 of the tubule. Intertubular space of the ob/ob testis appeared narrower and fewer Leydig
195 cells were found.

196 Seminiferous tubular cross-sectional area (Figure 2, top panel) and the percentage
197 of these tubules with sperm bundles (Figure 2, bottom panel) were subnormal in the
198 testes of ob/ob mice. . The number of spermatocytes per tubular cross section did not
199 differ between ob/ob mice and controls (Figure 2, middle panel; $P=0.062$).

200

201 **Apoptotic activity in testis**

202 Representative TUNEL stained testes from control and leptin deficient animals
203 are shown in Figure 3. Apoptotic activity within germ cells (particularly within pachytene
204 spermatocytes) was elevated in the testes of ob/ob mice compared to controls. The total
205 number of TUNEL positive germ cells per cross section and the number per seminiferous

206 tubule in cross section were significantly higher in ob/ob mice compared to controls
207 (Figure 4, top and bottom panel).

208 Microarray analysis on the testes of three ob/ob mice and two controls identified
209 nine pro-apoptotic and three anti-apoptotic genes that were expressed at a higher level (>
210 2-fold) in leptin deficient mice than controls (Figure 5). We were unable to identify any
211 apoptotic genes that were down-regulated in ob/ob mice. Among those pro-apoptotic
212 genes that were up-regulated were ones that code for peptidoglycan recognition proteins,
213 tumor necrosis factor receptor super family member-1A and 5 (TNFR 1 and 5),
214 sphingosine phosphate lyase 1, granzymes A and B, growth arrest and DNA damage
215 inducible 45 gamma, and caspases 7 and 9. The anti-apoptotic genes with higher
216 expression levels in leptin deficient mice included those coding for microphthalmia-
217 associated transcription factor, proviral integration site 2, and baculoviral IAP repeat
218 containing 4 (data not shown).

219 **Discussion**

220 The data from the current study support our previous findings that document
221 substantial abnormalities in testicular morphology and impaired spermatogenesis
222 associated with a leptin deficiency in the murine model (Bhat et al, 2003). In leptin
223 deficient animals, the testes and seminal vesicles were subnormal in size, Sertoli cells
224 exhibited substantial vacuolization, and Leydig cells had an abnormal fibroblastic
225 appearance. Within many of the seminiferous tubules of leptin deficient mice, cellularity
226 was reduced, there was condensation of germ cell nuclei, and an absence of mature
227 spermatozoa; distinct signs that the spermatogenic process had been arrested or greatly
228 impaired. However, as in our previous study (Bhat et al, 2003) the effects of the leptin

229 deficiency on testicular morphology were not diffuse throughout the testes of ob/ob
230 animals. There were regions within the testicular sections of leptin deficient animals
231 where tubular morphology and spermatogenesis appeared normal. The reasons for the
232 less than diffuse effect of the leptin deficit on testicular morphology are unknown
233 although it suggests that the impaired spermatogenic process in these animals results
234 from more than just a general gonadotropin deficiency. It is possible that localized
235 autocrine and/or paracrine mechanisms that support spermatogenesis are disturbed in
236 these animals as well.

237 The reduced germ cell numbers and the absence of mature spermatogenesis in
238 many seminiferous tubules suggested that the level of germ cell apoptosis might be
239 increased as a consequence of the leptin deficit. This theory was confirmed in the current
240 study using the *in situ* TUNEL assay for apoptotic activity. We were also able to identify
241 with microarray technology nine pro-apoptotic-related genes within the testes of leptin
242 deficient animals whose expression levels were significantly higher than in control testes.
243 These genes and their protein products are components of both the intrinsic and extrinsic
244 apoptotic pathway, suggesting that both pathways of programmed cell death are
245 accelerated within germ cells in the presence of a leptin deficiency.

246 To the best of our knowledge, the present study is the first to assess the impact of
247 a leptin deficiency on apoptotic activity within germ cells of the testis. The demonstration
248 that a leptin deficiency is associated with a greater than 3-fold increase in apoptosis
249 within germ cells (particularly pachytene spermatocytes) and impaired sperm production
250 supports the previous suggestion that in general the loss of germ cells in the testes occurs
251 primarily through programmed cell death (Bartke, 1995). The induction of a

252 gonadotropin and testosterone deficit in rats with GnRH antagonist treatment is
253 associated with increased apoptosis within preleptotene and pachytene spermatocytes and
254 spermatids (Sinha Hikim et al, 1995), and pachytene spermatocytes, dividing
255 spermatocytes and early round spermatids are especially susceptible to heat-induced
256 apoptosis (Lue et al, 1999). Thus, the data from the current study extends previous
257 findings to show that the impaired spermatogenic process in the leptin deficient mouse is
258 associated with a quantifiable elevation in germ cell apoptosis, and are consistent with the
259 previous observations that early germ cell stages are especially vulnerable to increased
260 programmed cell death during adverse conditions.

261 The present study is also the first to attempt to identify (via microarray)
262 apoptosis-related genes whose expression levels within the testis are altered by a leptin
263 deficiency. Of the nine genes identified, three are components of the extrinsic apoptotic
264 pathway and five are from the intrinsic apoptotic pathway. The final gene identified
265 encodes for caspase-7, one of the executioner genes upon which the intrinsic and
266 extrinsic pathways converge in the apoptotic cascade.

267 One of the genes associated with the extrinsic pathway that was highly expressed
268 (increased 5.1 fold over control levels) in the testis of the ob/ob mouse encodes for the
269 peptidoglycan recognition protein (PGRP). PGRPs form stable complexes with heat
270 shock protein 70 (Hsp70) that is synthesized during the meiotic phase of spermatogenesis
271 and is abundantly expressed in pachytene spermatocytes (Eddy, 1994; Dziarski 2004;
272 Sashchenko et al., 2004). Neither the PGRP's nor Hsp70 is cytotoxic alone, but when
273 complexed together they induce apoptotic cell death in several tumor cell lines
274 (Sashchenko et al, 2004).

275 Expression levels of the two genes that encode for TNFR1 and 5 were also
276 increased above control values in the testes of leptin deficient mice. TNFR1 is known to
277 be involved in the extrinsic cell death pathway (Baker and Reddy, 1998). Its ligand, TNF-
278 α , binds to TNFR1 activating caspases. Murine pachytene spermatocytes and round
279 spermatids express TNF- α mRNA, and the latter are also capable of secreting TNF- α
280 bioactivity *in vitro* (De et al, 1993). Since our gene array study was performed using
281 whole testes it is not possible to ascertain the exact cell types over-expressing TNF
282 receptors.

283 Expression levels for two genes that code for two components (granzyme A and
284 B) of the intrinsic apoptotic pathway were also elevated in the testes of ob/ob mice.
285 Granzymes are serine proteases that serve as effector molecules for cytotoxic T
286 lymphocytes and natural killer cells (Yamada et al, 2003). The combined action of these
287 molecules is known to initiate apoptosis of target cells. Granzyme A mediates
288 glucocorticoid-induced apoptosis in 697 leukemia cells by increasing caspase-3 activity
289 perhaps upstream of Bcl-2 signaling (Yamada et al, 2003). Granzyme B appears to
290 trigger apoptosis by directing the pro-apoptotic molecule, Bid, to the mitochondrial
291 membranes facilitating cytochrome c release (Ida et al, 2003). The present study appears
292 to be the first to report granzyme expression in the murine testis.

293 Sphingosine-1-phosphate lyase (SPL), an enzyme that catalyzes the cleavage of
294 intracellular second messenger sphingosine-1-phosphate (Maceyka et al, 2002), causes
295 ceramide accumulation and/or sphingosine phosphate depletion leading to sustained
296 cytochrome release and increased apoptosis in HEK293 cells (Reiss et al, 2004). During
297 male germ cell apoptosis, ceramide levels increase before appearance of caspase-3

298 activation and DNA fragmentation, and germ cell death can be inhibited by exogenous
299 administration of sphingosine phosphate to the cultured human seminiferous tubules
300 (Suomalainen et al, 2003). In the present study, expression of the gene encoding SPL
301 (another component of the intrinsic apoptotic pathway) was elevated in the testes of
302 ob/ob mice suggesting that SPL may play a role in mediating the enhanced level of germ
303 cell apoptosis in leptin deficient animals.

304 Expression of the growth arrest and DNA damage 45 (GADD45) gene was also
305 increased above control levels in the testes of ob/ob mice suggesting that GADD45 may
306 participate in the apoptosis and/or DNA repair occurring in the germ cells of leptin
307 deficient mice. Exposure to ionizing radiation induces the transcription of GADD 45
308 which inhibits proliferation and stimulates DNA excision repair in mammalian cells
309 (Fornace et al, 1989; Hollander et al, 1993). Phorbol ester treated MCF-7 breast cancer
310 cells expressed GADD45 before the onset of apoptosis (De Vente et al, 1995). The
311 expression of GADD45 in brain regions of rats following excitotoxic lesion correlated
312 with DNA fragmentation as detected by TUNEL staining (Hughes et al, 1996).

313 We also found increased levels of expression of genes coding for caspase 7 and 9
314 in the testes of leptin deficient mice. Caspase 9 is an initiator of activation of the caspases
315 that act as executioners of apoptotic processes (Johnson and Bridgham, 2002). Caspase 7
316 is one of the executioners of apoptosis that is thought to play a role in ovarian follicular
317 atresia (Matikainen et al, 2001; Johnson and Bridgham, 2002). Our findings in the current
318 study of increased germ cell apoptosis in conjunction with elevated gene expression
319 levels for caspase 7 and 9 suggest that these genes are integral components of the cascade
320 that results in increased germ cell death within the testis of mice with a leptin deficit.

321 The expression of three anti-apoptotic genes was also up-regulated in the testis of
322 leptin deficient mice. Among these were proviral integration site 2 (Pim-2),
323 microphthalmia-associated transcription factor (Mitf) and baculoviral IAP repeat
324 containing 4 (BIRC4). Pim-2 is a member of a small family of oncogenic serine/threonine
325 kinases and provides long-term resistance to a variety of apoptotic stimuli (Fox et al,
326 2003), and has been shown previously to be expressed in the spermatocytes and
327 interstitial tissue of normal human testes (Baytel et al, 1998). Mitf belongs to a family of
328 transcription factors and is expressed in spermatogonia, spermatocytes and round
329 spermatids of mouse testis (Hodgkinson et al, 1993; Saito et al, 2003). The physiological
330 significance of Mitf expression in male germ cells is not known. BIRC4 belongs to the
331 baculovirus IAP repeat-containing protein family known as the inhibitors of apoptosis
332 (Wang et al, 2004). The reasons why these anti-apoptotic genes are up-regulated in the
333 testes of ob/ob mice that show elevated apoptosis are unknown, but these genes may be
334 attempting to serve as protectors of germ cells during accelerated programmed cell death.

335 A question that remains unresolved is the mechanism by which the leptin deficit
336 adversely alters germ cell production. Gonadotropins are known to be anti-apoptotic
337 agents (Tapanainen et al, 1993). It appears appropriate, therefore, to suggest that the
338 gonadotropin deprivation may be responsible for the increased germ cell loss in the leptin
339 deficient model. Alternatively, the leptin deficiency may directly alter the spermatogenic
340 process since testicular tissue expresses leptin receptors (El-Hefnawy et al, 2000; Caprio
341 et al, 2003), and leptin can directly alter testicular function *in vitro* (Tera-Sempere et al,
342 1999; Giovambattista et al, 2003).

343 Leydig cells were morphologically abnormal and fewer and seminal vesicles were
344 smaller in ob/ob animals. This is suggestive of under-androgenization, but surprisingly
345 total serum testosterone concentrations were in the normal range. This is in agreement
346 with an earlier study from our laboratory (Bhat et al, 2003). The reasons for these
347 seemingly conflicting data are unclear. The normal total testosterone levels in leptin
348 deficient mice were unexpected because these animals have reduced circulating
349 gonadotropin levels and impaired GnRH secretion (Swerdloff et al, 1976; Batt et al,
350 1982). This could be due to the fact that testosterone is secreted in a pulsatile and
351 circadian pattern and that by only measuring the hormone at one time point, we may have
352 missed a significant reduction at another time point that resulted in a reduced 24 h
353 secretory rate. Another possible explanation is that the leptin deficient mouse produces a
354 binding protein that lowers free biologically active testosterone while reducing the
355 clearance rate of the androgen. Related to this issue, we have found a similar
356 phenomenon in the ob/ob female mouse where total serum estradiol and progesterone
357 levels are actually elevated above control levels but uterine weights are subnormal
358 (Olatinwo et al, unpublished data). In any case, this is an interesting issue that deserves
359 further study.

360 In conclusion, we have identified a group of genes via microarray technology that
361 may play a prominent role in mediating the increased germ cell apoptosis and impaired
362 sperm production exhibited by leptin deficient mice. These genes are components of both
363 the extrinsic and intrinsic pathway of programmed cell death. Future studies will be
364 needed to determine whether the effects of the leptin deficiency on the spermatogenic

365 process are a consequence of the hypogonadotropic status of these animals or whether
366 leptin can directly modulate spermatogenesis at the level of the testis.

367

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482 **Figure Legends**

483 **Figure 1:** Representative photomicrographs of hematoxylin and eosin staining of the
484 testes cross-sections from control (Panel A) and leptin-deficient (Panels B, C and D)
485 mice. Panels B and C illustrate the reduced cellularity, condensation of germ cell nuclei
486 (short white arrows) as well as Sertoli cell vacuolization (long arrows) common to the
487 testes of ob/ob mice. However, in some regions of the testis of ob/ob mice,
488 spermatogenesis appeared to be normal (Panel D). Magnification: X200.

489 **Figure 2:** Mean \pm SEM of cross-sectional seminiferous tubule area, number of
490 spermatocytes per tubule cross section and percentage of tubule per cross-section with
491 sperm bundles in the testes of ob/ob and control mice. * Significantly different from
492 control values, $P < 0.05$.

493 **Figure 3:** Representative photomicrographs of TUNEL stained testicular cross-sections
494 from a control (A and C) and two ob/ob (B and D) mice. Whereas the testes of control
495 mouse showed only a few TUNEL positive cells, testes of ob/ob mice exhibited more
496 TUNEL positive germ cells, particularly the pachytene spermatocytes. Magnification, A
497 and B: X100, C and D: X200.

498 **Figure 4.** Mean \pm SEM of total number of TUNEL positive germ cells per 10 random
499 fields (top panel) and number of TUNEL positive germ cells/seminiferous tubule (bottom
500 panel) in the testes cross-sections of ob/ob and control mice. * Significantly different
501 from control values, $P < 0.05$.

502 **Figure 5:** Fold increases in the expression levels of pro-apoptotic-related genes in the
503 testes of ob/ob mice versus the controls as determined by microarray analysis. PGRP =
504 peptidoglycan recognition protein; TNFRSF1a = tumor necrosis factor receptor

505 superfamily member 1a; TNFRSF5 = tumor necrosis factor receptor superfamily member
506 5; Gzma = granzyme A; Gzmb = granzyme B; Sgpl1 = sphingosine phosphate lyase 1;
507 Gadd45 = growth arrest and DNA damage inducible 45 gamma; Casp9 = caspase 9;
508 Casp7 = caspase 7. Extrinsic and Intrinsic refer to the Fas-FasL/TNF-TNFR and Bcl2-
509 Bax system pathways of apoptosis, respectively.

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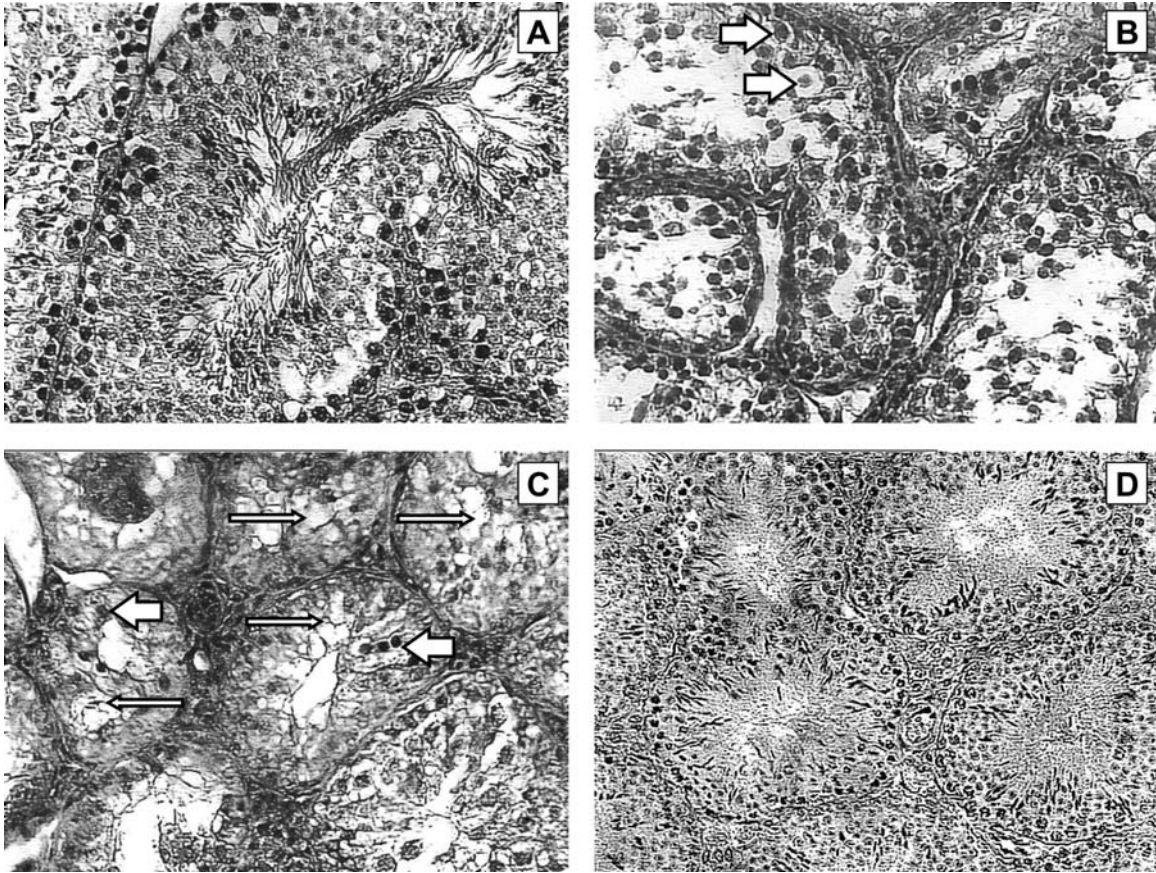
526 Table 1. Effect of a leptin deficiency on mean \pm SEM of body weight, weight of testes
 527 and seminal vesicles, and serum testosterone and leptin concentrations.

	Control Mice	Leptin Deficient Mice
Body Weight (g)	27.9 \pm 0.6	62.5 \pm 0.9*
Weight of Testes (mg)	205.6 \pm 4.9	174.3 \pm 13*
Weight of Seminal Vesicles (mg)	282.4 \pm 17.6	197.3 \pm 30.1*
Serum Testosterone (ng/ml)	0.62 \pm 0.01	0.67 \pm 0.07
Serum Leptin (ng/ml)	2.1 \pm 0.22	0.21 \pm 0.04*

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 529 Values marked with an asterisk are significantly different from control values ($P < 0.05$).

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530 Figure 1.



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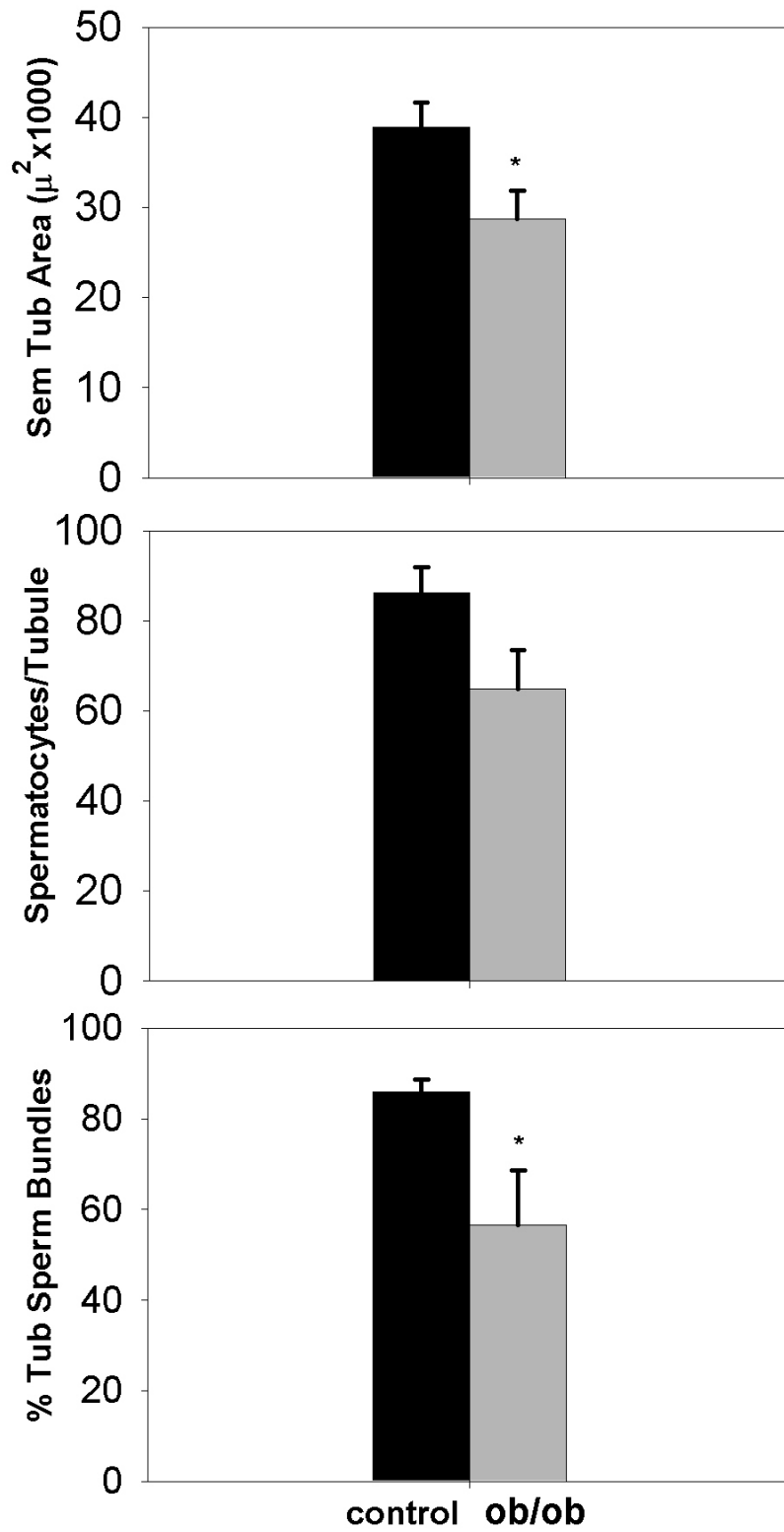
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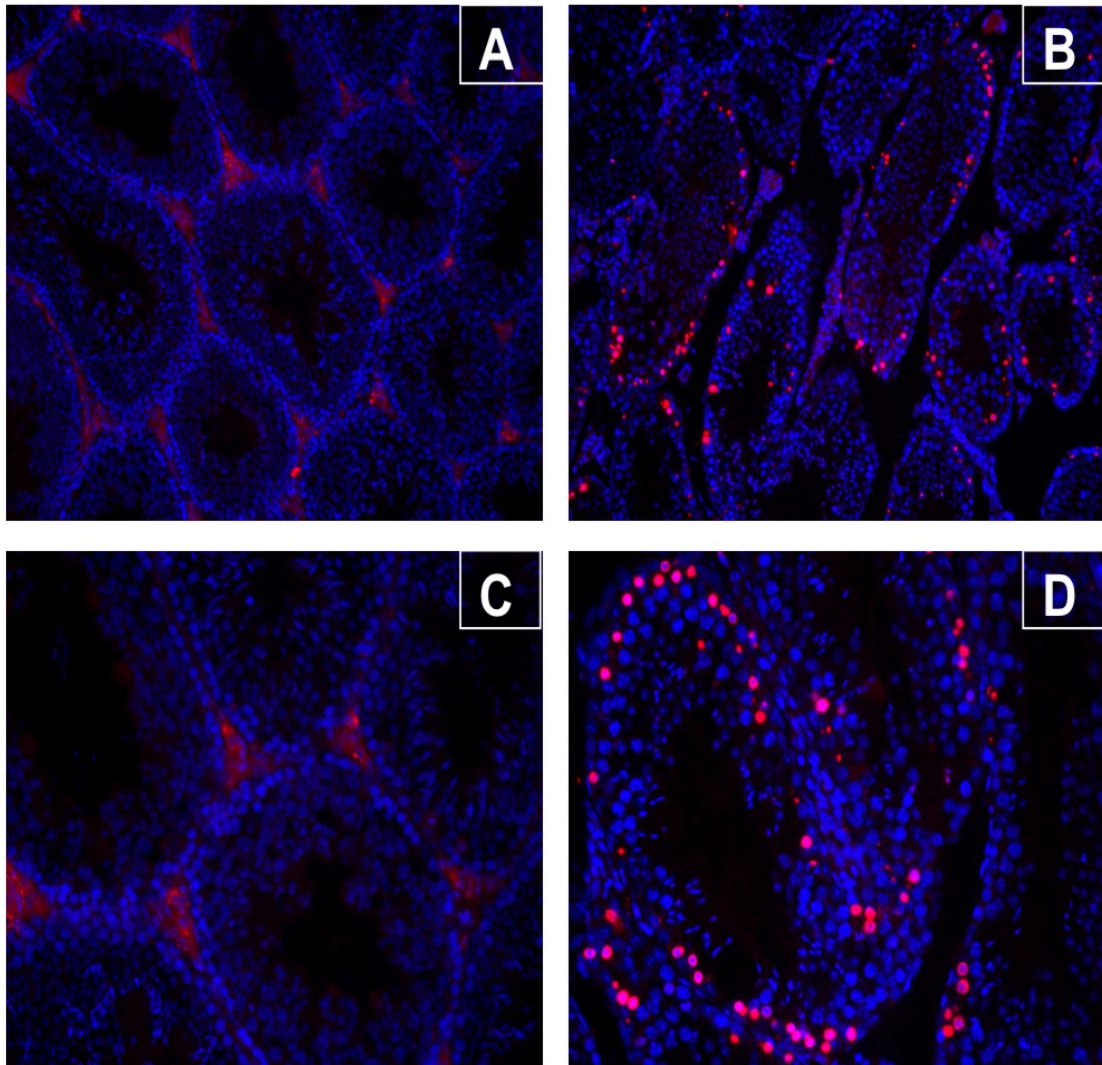
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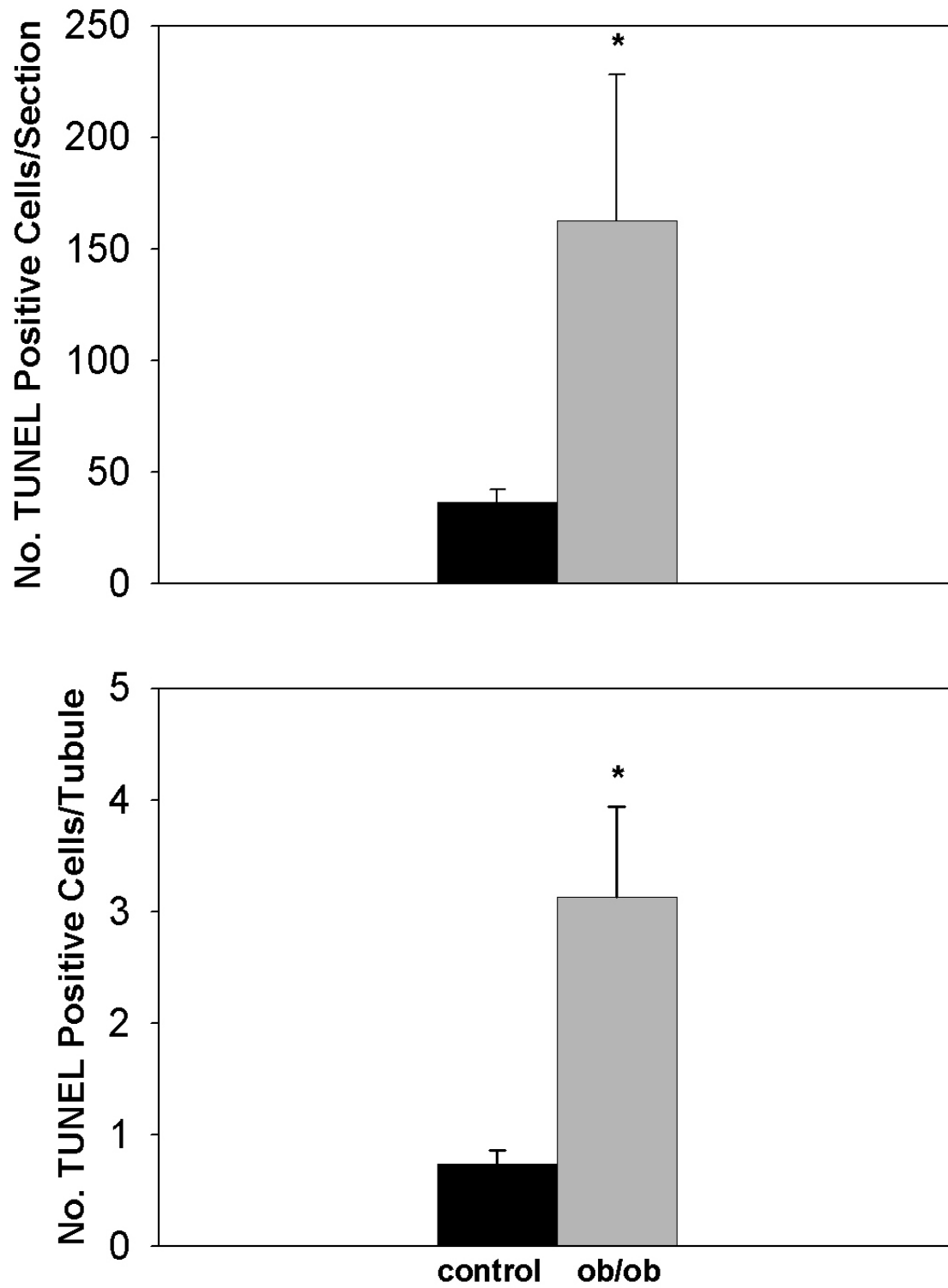
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546 Figure 3.

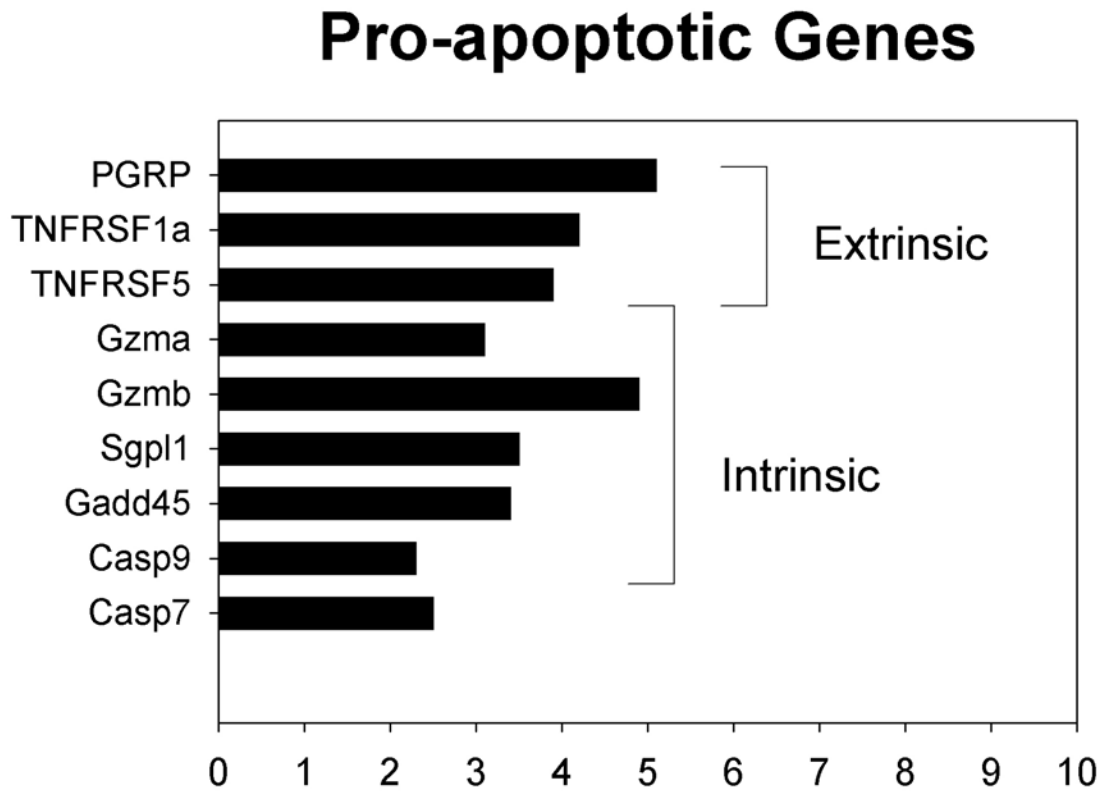


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